PROJECT NUMBER: 1904
PROJECT TITLE: Tobacco Physiology and Biochemistry
PROJECT LEADER: H. Y. Nakatani
PERIOD COVERED: February, 1988

I. LOW NICOTINE STUDY

A. **Objective**: To investigate the biochemistry of the nicotine biosynthetic pathway at putrescine N-methyltransferase (PMT) and specifically to isolate PMT from tobacco root extracts.

B. **Status**: The harvest of the roots from the group nine plants was staggered to examine the yield post-topping since the root mass from the last two groups was quite small. The extraction of roots from group nine, one week (PM53) and two weeks (PM54) post-topping was completed to the 40-65 % ammonium sulfate stage and treated to allow application to the first equilibrated phenyl-Sepharose column (1).

Another octyl-Sepharose column was investigated using a NaCl rather than ammonium sulfate gradient. This column did not enhance the resolution over the phenyl-Sepharose columns currently in use (3,4). Hydroxyapatite chromatography was also examined further. Binding characteristics were determined; however, substantial loss in PMT activity will likely preclude the use of this support medium in the purification scheme (2).

Multiple PMT-active samples were run on the 1 X 40 cm Sephadex G-100 column (minus glycerol) to obtain material for the Hazleton antibody contract. Pooled fractions were concentrated to approximately 22 ug/150 ul (total 5.8 ml) (6) which had a specific activity of 7.3×10^4 units per mg protein (3). [One unit equals 1 nmole of SAM (^{14}C-S-adenosylmethionine) 30 min of reaction time.] The ammonium sulfate fraction had a specific activity of 1.8×10^3 units per mg protein (3).

A commercially prepacked Sephacryl S200HR (Pharmacia) gel filtration column (1 liter bed volume) was set-up and is being equilibrated and calibrated for use (6). The PMT-active fractions were pooled from the first phenyl-Sepharose column and precipitated with ammonium sulfate (65 % saturation) to be applied to this column.

Several ELISA (enzyme linked immunosorbent assay) experiments, using a rabbit antibody against phenylethanolamine N- methyltransferase (PNMT-Ab)) were conducted to compare the color reaction (alkaline phosphatase) with PMT and PNMT samples (total protein concentration from 1-4 ug). A twenty to forty-fold enhancement of the color reaction was observed with the authentic PNMT and the PNMT-Ab over the PMT samples and the generic antibody (7). A miniblotting device to determine the cross-reaction of antibody(ies) against proteins which have been transferred to nitrocellulose and PVDF (polyvinylidene difluoride) paper (Western blot) is also being examined (7). Preliminary background absorption problems are being addressed. This device will allow use of

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approximately 50-75 ul of antibody solution versus many milliliters for the classical methods, a concern especially with monoclonal antibody production. A magnetic bead (MagniSort bead, DuPont) bound with an antibody against rabbit IgG is also being examined and shows promise for our screening efforts (7).

The technique of photoaffinity labeling of PMT with ^{3}H-S-adenosyl-methionine (SAM), using UV irradiation is currently under investigation (8). Our initial examination has been conducted with PNMT. The conditions for incorporation appear to be satisfactory and PMT samples will be exposed to a similar treatment. Controls were run with either excess SAM or S-adenosylhomocysteine. ^{3}H-S-adenosyl-methionine assays have been conducted as necessary to support the PMT purification effort (3).

C. **Plans**: The next group of hydroponically-grown tobacco plants (group 10) will be topped sometime near mid-March. The roots will be harvested thereafter and extracts will be prepared. The purification of PMT will continue. Studies will continue with various chromatographic media. Sequential samples will be applied to the gel filtration column (1x40 cm). The large-scale, commercially prepacked, Sephacryl gel filtration column will also be tested with PMT containing fractions from the initial phenyl-Sepharose column. Antibody binding studies will continue using MagniSort-beads, Western blots and microtiter plates. ^{3}H-SAM labeled PNMT and PMT samples will be examined by SDS-PAGE.

D. **References**:

1. Sherwood, K. R. Notebook No. 8545.
2. Malik, V. S. Notebook No. 8542.
3. Nakatani, H. Y. Notebook No. 8384
4. Dunn, R. L. Notebook No. 7899.
5. Sykes, A. Notebook No. 8526.
6. Yu, T. Notebook No. 8381.
7. Mooz, E. D. Notebook No. 8599.
8. Davies, B. D. Notebook No. 8005.

II. ALTERNATE HUMECTANTS (PG/G-FREE SHEETS/CIGARETTES) (1)

A. **Objective**: To find a plasticizer/humectant system that provides acceptable sheet materials without glycols (PG/G).

B. **Results**: Subjective evaluation was made of the 100% cigarettes from production plant RL and RCB sheets containing K-pp in place of PG-pp. No significant difference was found between the test and the control cigarettes. Subjective evaluation was made of the 100% cigarettes made from PG/G-free RCB handmade sheets produced in the Pilot Plant. A level of 4% isosweet substituted for PG/G gave the best results.

C. **Plans**: Memos will be issued by Flavor Development summarizing the data from the subjective evaluations described above. Subjective evaluation will be made of the 100% cigarettes from PG/G-free RL

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sheets made in the Pilot Plant. Plans will be made to produce PG/G-free RL and RCB feedstocks at the production plants in March.

D. **References**:

1. Mooz, E. D. Notebook No. 8599.

III. ALTERNATE HUMECTANTS (GLYCERINE-FREE SHEET/CIGARETTES) (1)

A. **Objective**: To provide acceptable glycerine-free (G-free) RLTC, RL150B, and RCB sheets and G-free cigarettes for the European market.

B. **Results**: Subjective evaluation was made on the 100% cigarettes from production plant RL and RCB G-free sheets. The RL products were found to be similar, but the RCB models were considered "atypical". A memo was issued summarizing the results (2). As a result of these subjective tests, G-free RCB was remade at the BL plant. G-free, cased ET and ESB were also made.

C. **Plans**: A POL test is planned with a G-free Marlboro type cigarette and a Marlboro in which the recons will be made with no class tobacco to be tested versus a control Marlboro cigarette.

D. **References**:

1. Mooz, E. D. Notebook No. 8599.
2. Spielberg, H. L. Reconstituted Tobacco without Glycerine. Memo to Mr. D. Knudson; 1988 February 2.

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